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Sequence determination of a peptide fragment from electric eel acetylcholinesterase, involved in the binding of quaternary ammonium

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Specific photoaffinity labelling of purified electric eel acetylcholinesterase by ^{3}H-labelled *p*-(*N,N*-dimethylamino)benzenediazonium fluoroborate allows the identification of a labelled peptide fragment which is described as being involved in the binding of quaternary ammonium ions on this enzyme. Denaturation and proteolytic cleavage of the inactivated enzyme gave a mixture of peptide fragments. The purification of one labelled fragment, containing over 15% of the radioactivity incorporated in the enzyme, led to the following sequence: Gly-Ser-X-Phe. The relatively low amount of this tetrapeptide did not allow us to determine the nature of the labelled residue X.

Photoaffinity labeling *Aryldiazonium salt* *Acetylcholinesterase* *Quaternary ammonium binding site* *Peptide sequence*

1. INTRODUCTION

The electric organ of *Electrophorus electricus* contains different molecular forms of acetylcholinesterase [1] (acetylcholine acetylhydrolase, EC 3.1.1.7). The native asymmetric forms can be obtained in a fairly highly purified state [2] and in reasonable quantities. This purified material has been used for irreversible photoaffinity labelling experiments [3]. Successful labelling can ultimately contribute to the knowledge of the primary structure of the target binding site by determining the sequence of a peptide fragment to which the label is covalently bound.

Some aryldiazonium derivatives have been shown to be good photoaffinity labels for AChE [4] leading to a light-induced irreversible inhibition which can be protected by tetraalkylammonium derivatives. Among these aryldiazonium derivatives DDF appeared to be particularly attractive for the following reasons: (i) in the absence of light it is a good competitive inhibitor of this enzyme $K_i = 2 \times 10^{-5}$ M; (ii) in the presence of light it becomes a very efficient irreversible marker according to the hyper-reactivity of the photogenerated aryl cation [5]; (iii) energy-transfer-induced photoaffinity labelling is effective with this reagent on AChE leading to specific irreversible alkylation [6]; finally, this reagent can easily be synthesized in a radioactively labelled state.

Dedicated to Professor Guy Ourisson on the occasion of his 60th birthday

Abbreviations: AChE, acetylcholinesterase; DDF, *p*-(*N,N*-dimethylamino)benzenediazonium fluoroborate; PTH, phenylthiohydantoin; PTZ, phenylthiazolinone; DABITC, *p*-(*N,N*-dimethylamino)azobenzene isothiocyanate; DABTH, *p*-(*N,N*-dimethylamino)azobenzene thiohydantoin

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The structure determination of a small peptide alkylated by radioactive DDF is described here. This peptide fragment belongs likely to the quaternary ammonium binding site of AChE.

2. EXPERIMENTAL

2.1. *Enzyme inactivation*

AChE was purified in 6 different batches from electric organs of electric eel by adapting a described procedure [2]. Purified enzyme (1 mg/ml) was inactivated by a 10^{-4} M solution of [^{3}H]DDF (0.2 Ci/mmol) in energy transfer photolabelling conditions as described in [6].

After dialysis the labelled enzyme was denatured by an 8 M urea–Tris-HCl (pH 8.5) buffered solution and carboxymethylated by excess iodoacetamide after β-mercaptoethanol treatment. There was no loss of radioactivity during this process.

2.2. *Proteolytic cleavage*

Successive proteolytic treatments were performed under the following experimental conditions.

Trypsin: 2 h incubation at 37°C of a mixture of protein (1 mg/ml) in 1% ammonium bicarbonate and trypsin (1%, w/w).

Staphylococcus aureus protease: two successive incubations at 37°C (3 and 15 h) of a mixture of peptides (1 mg/ml) in 1% ammonium bicarbonate and the protease (15%, w/w).

Pepsin: two successive incubations at 37°C (3 and 15 h) of a mixture of peptides (1 mg/ml) in 10% acetic acid and pepsin (2.5%, w/w).

2.3. *Peptide purifications*

After Sephadex G-15 gel filtration in 10% acetic acid the collected fractions were chromatographed by HPLC (Waters model 660) using a C_{18} Beckman or a C_{18} Bio-Rad column (ODS 5 μm). The different elution gradients are shown in fig.2.

2.4. *Amino acid composition*

Amino acid analyses were carried out using a Durrum D500 amino acid analyser. Peptides dissolved in 6 N HCl containing 0.02% β-mercaptoethanol were hydrolysed in vacuo at 110°C for 2 h. Methanesulfonic acid hydrolysis showed the absence of Trp residues in the analysed peptides.

2.5. *Sequencing*

Liquid-phase sequencing was done using a Beckman spinning-cup sequencer (model 890C). The collected ATZ amino acids were converted after 30% trifluoroacetic acid treatment (80°C, 10 min) to the corresponding PTH amino acids which were analysed by HPLC on a Zorbax-CN column.

2.6. *DDF photodecomposition in N-ethylacetamide*

A stirred solution of DDF (10 mg) in *N*-ethylacetamide (10 ml) was irradiated for 7 min under argon by a Philips HPK 125W lamp. This solution was quenched with sodium acetate (pH 5) buffered solution. After 1 h the pH was brought to 7 and the solution extracted with ethyl acetate.

After preparative thin-layer chromatography (ethyl acetate/hexane, 1:1, as eluent) the compounds were identified by comparison with synthetic samples. A quantitative analysis of the mixture was done by gas chromatography (Girdel model 300 on a 10% Carbowax 20M column).

3. RESULTS

3.1. *Photochemical reactivity of DDF towards an amide*

We checked the photochemical reactivity of DDF towards *N*-ethylacetamide as a model for a peptide bond. This reaction led to almost quantitative *N*- and *O*-alkylation of the amide bond in a ratio N/O = 1:9 by referring to the proportions

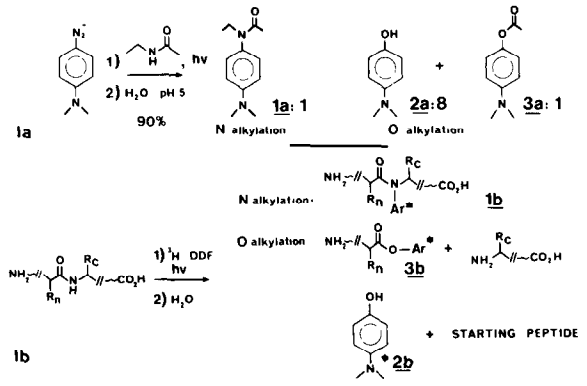

Fig.1. (a) Analysis of the photochemical reaction of DDF with *N*-ethylacetamide. (b) Possible photochemical reaction of DDF with a peptide bond.

of isolated and identified compounds **1a–3a** after hydrolysis at pH 5 (fig.1a). The hydrolysis of the *O*-alkylated species gave two derivatives, the phenol **2a** and the acetate **3a** in an 8:1 ratio. More acidic hydrolysis conditions tend to lower this ratio [7].

If such an alkylation reaction with radioactive DDF were to occur on a peptide backbone the following result would be expected (fig.1b): the *N*-alkylation process will lead to the formation of the radioactive *N*-aryl peptide **1b**. The *O*-alkylation process leads in part to the recovery of the initial peptide together with the radioactive phenol **2b** as well as to the cleavage of the initial peptide into two peptide fragments one of them being a radioactive C-terminal aromatic ester **3b**.

3.2. *Isolation and purification of a peptide fragment from electric eel AChE photoalkylated by [^{3}H]DDF*

Six different batches of over 90% pure AChE were prepared from electric organs of *E. electricus*. Each preparation was separately alkylated with [^{3}H]DDF under energy transfer photolabelling conditions. The use of 10^{-4} M label allowed 90% irreversible inactivation of the enzyme. The stoichiometry of irreversible labelling was determined on each batch and we found an average of 1.05 ± 0.10 molecules of [^{3}H]DDF covalently bound per enzyme active site.

Denaturation and carboxymethylation of the alkylated enzyme led to complete recovery of the radioactivity. According to the amino acid composition of this enzyme [8] several proteolytic enzymes could be used to cleave the polypeptide chain. The successive treatment of the alkylated polypeptide chain with trypsin, *S. aureus* protease and pepsin gave a fingerprint where the radioactive peptides are well separated from the unlabelled fragments. The radioactive peptides show neutral and non-polar properties. Gel filtration (Sephadex G-15) of the crude proteolytic fragments gave 3 main peaks from which the lighter fractions which represent 40% of the initial radioactivity showed an interesting HPLC chromatogram in terms of *A* vs radioactivity profiles (fig.2, top).

The different purification steps of the radioactive peptide fragment are summarised in fig.2. The overall radioactivity recovery in this purified peptide is about 16% of the crude hydrolysis mixture (taking into account the yield of each step).

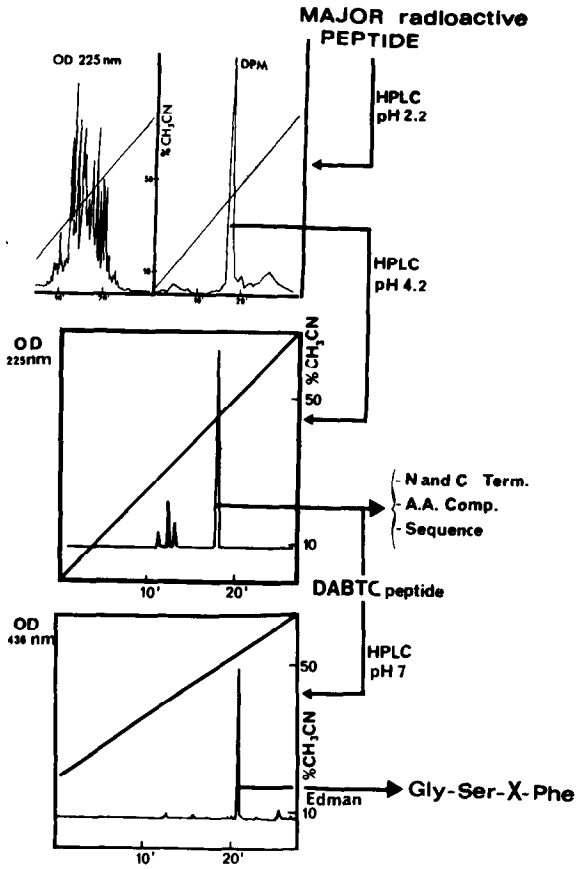

Fig.2. C_{18} reverse-phase HPLC purification steps of a peptide fragment labelled by [^{3}H]DDF (see section 2). pH 2.2: trifluoroacetic acid 0.5% in water/CH_3CN. pH 4.2: ammonium formate buffer/CH_3CN. pH 7: sodium phosphate buffer containing β-mercaptoethanol (5%, v/v)/CH_3CN.

3.3. *Sequence determination of the labelled peptide fragment*

The N- and C-termini of the radioactive peptide fragment were each determined on 1 nmol partially purified material (fig.2).

Cleavage with either carboxypeptidase A or Y showed a single C-terminal residue identified as phenylalanine. Dansylation of the peptide gave a unique N-terminal amino acid identified as glycine. In both determinations we observed an apparent purity of the peptide which was in partial contradiction to the amino acid composition (table 1). In fact, the relatively small amount of material

Table 1

Amino acid composition of the partially purified peptide fragment modified by [^{3}H]DDF

Asp	0.35
Thr	0.15
Ser	0.95
Glu	0.35
Pro	0.15
Gly	1.25
Ala	0.2
Met	0.15
Ile	0.1
Phe	0.7
Lys	0.1

Residues are expressed as mol per mol peptide

(1 nmol) which was used in these N- and C-terminal determinations might explain this discrepancy. A sequence determination on this material by Edman degradation using the automated liquid-phase 'spinning cup' methodology gave the tentative sequence Gly-Ser-X-Phe. The residue X obtained at the third step did not correspond to any known PTH amino acid derivative and it was the only sequencing step which contained radioactivity (about 65% of the content injected into the sequencer). Therefore, X should correspond to an amino acid residue which has been modified by photoalkylation with DDF. Purification of this peptide was necessary to confirm the proposed sequence. This was realised by coupling 5 nmol of the remaining peptide to the coloured DABITC reagent [9]. HPLC of the DABTC peptide obtained makes use of the easy detection in the visible region.

The N-terminal determination by DABTH amino acid analysis showed the presence of two residues: the expected glycine as the major peak and aspartic acid with about 35% of the relative intensity towards glycine. The sequencing of the remaining tripeptide by automated liquid-phase methodology and HPLC analysis on the PTH amino acids confirmed the proposed sequence.

3.4. *Tentative identification of X by mass spectrometry*

Several trials on the identification of the modified residue by mass spectrometry failed. These attempts were made on the X-PTH derivative collected during the sequencing process by NH_3 chemical ionisation methodology. Several attempts have been made and there was no possible fragmentation correlation with spectra obtained from different synthetic PTH amino acids injected under comparable conditions. This was due mainly to the very low signal/noise resolution. A final trial on the tripeptide Ser-X-Phe by fast atom bombardment methodology also did not succeed.

4. DISCUSSION

Successful labelling of a target binding site by photoaffinity labelling necessarily involves the photogenerated reactive species reacting instan-

Table 2

Active site region from cholinesterases

Eel AChE [11]
Gly Gly Glu Ser Ser Glu Gly Ala Ala Gly

Torpedo AChE [17]
Thr Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Ala Ser Val Gly Met His Ile Leu Ser Pro Gly Ser Arg

Equine pseudocholine esterase [18]
Phe Gly Glu Ser Ala Gly Ser Ala Ala

Human serum ChE [19]
Ser Val Thr Leu Phe Gly Glu Ser Ala Gly Ala Ala Ser Val Ser Leu His Leu Leu Ser Pro Gly Ser His Leu Thr Ser Arg

taneously with any residue present in its surroundings. Aryl cations generated photochemically from aryldiazonium salts are highly energetic species [5]; they are among the few intermediates which are able to react with molecular nitrogen [10]. Consequently one expects several residues which are in the vicinity of the label to become labelled in such a process.

The tetrapeptide we have isolated and partially identified is one among other labelled peptides (it represents about 16% of the total radioactivity incorporated in the protein). The very low amount of material did not allow us to determine the nature of the modified residue X, nevertheless several comments can be made. Very likely the alkylation reaction did not occur on the peptide backbone according to the total recovery of radioactivity after denaturation of the protein (fig.1b). We can also exclude the formation of an aromatic ester by reaction with a carboxylic acid side chain from Asp or Glu according to the high stability of the newly formed chemical bond towards acidic or basic hydrolysis reactions.

The knowledge of the primary structure of electric eel AChE should allow to position this tetrapeptide in the sequence. It is interesting to note that this peptide is different from the decapeptide known around the reactive serine of electric eel AChE [11] or other cholinesterases' active site sequences (table 2). However, the peptide fragment Gly-Ser-Phe-Phe (positions 328–331) is found in the sequence of *Torpedo california* AChE which has been published recently [12]. But one has to keep in mind that there might be major differences in the AChE sequences according to their origin.

Interaction of competitive inhibitors like DDF with AChE occurs very likely at a quaternary ammonium binding site. Taking into account the hyper-reactivity of the photogenerated aryl cation and a stoichiometry of irreversible incorporation close to unity it can be concluded that the labelled residue is part of this binding site. Unlike most other organic cations the quaternary ammonium cannot form hydrogen bonds and therefore the forces controlling its binding to the enzyme must be largely a function of the distribution of charge within the entire cation. Crystal structure analysis of quaternary ammonium salts containing tartrates as counterions [13] shows a relatively even complexation distribution of the cation through several oxyanion and hydroxyl groups within a distance of 5 Å from the nitrogen atom. The binding of quaternary ammonium salts including acetylcholine by synthetic macrocyclic receptors has been described recently [14] and does in fact make use of a hydrophobic cavity surrounded by carboxylate residues. Such a general description of this binding site combines the concept of the 'anionic site' [15] with the 'trimethyl site' [16] which emphasises the hydrophobic nature of this binding site. Our tetrapeptide is hydrophobic and does in fact contain at least one hydroxylic side chain residue (Ser). It can be noted that all the sequences around the active site of cholinesterases (table 2) also show a pronounced hydrophobic character as well as the presence of many Ser and Thr residues.

More information is of course necessary to reconstitute the three-dimensional picture of such a binding site starting with the publication of the primary sequence of the protein. In such a context the identification of several peptide fragments which contribute to the binding of a ligand will be very helpful.

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